measured pursuant to the present invention. Applicant respectfully traverses the Examiner's rejection.

It is respectfully submitted that the claimed invention is enabled. The invention as claimed relates to a method for measuring the total amount of intact hCG and ITA or the total amount of intact hCG and ITA plus the free beta form of hCG in a biological sample from a patient at risk for gestational trophoblastic disease or quiescent gestational trophoblastic disease as claimed and determining the amount of total intact hCG plus ITA or total hCG plus beta core plus ITA with the amount of ITA measured *using an immunoassay which determines the selective binding of monoclonal B152 to ITA* in the sample such that a diagnosis of gestational trophoblastic disease or quiescent gestational trophoblastic disease may be made. It is respectfully submitted that the present invention is enabled.

The measurement of the amount of intact hCG,, ITA and beta core hCG as defined in the specification in a urine, serum or plasma sample is well known in the art. The approach to measuring this amount may be through an immunoassay, or other commercially available hCG tests. Indeed many commercial assays measure same. This is discussed in significant detail in the specification *inter alia,* at page 7, fourth full paragraph of the specification, as well as on pages 8-10. Approaches for measuring hCG in biological samples have been known for years and are well documented and routine in the art. The measurement of ITA as defined in the specification (containing both N-glycosyl linkages and O-glycosyl linkages as indicated in the specification) is also well known in the art. Analysis can be performed by any number of techniques as described in the present application at pages 8-10 and in particular, in an immunoassay using the B152 antibody which is specific for ITA (and the O-glycosyl linkages of ITA as explained in the Valmu, et al. paper and the enclosed paper of Birken). Thus, the present invention relates to the measure of ITA as opposed to the N-glycosylated variant measured by Kobata and provides a well-known method available in the art including a specific monoclonal antibody B152 which is specific for ITA.

Thus, the present invention provides all of the well-known methods for measuring amounts of intact hCG, beta hCG and ITA in urine, serum and plasma samples from a patient. The remaining steps are also well-known and are enabled. It is respectfully submitted that the instant claims are clearly enabled.

Turning to the Examiner's rejection of claims 12 and 47 as being non-enabled (section 6 on pages 5-6), it is respectfully submitted that with the amendment to claim 12 and in particular, to the fact that the patient to be diagnosed is a patient *previously diagnosed as having quiescent gestational trophoblastic disease or previously treated for an invasive gestational trophoblastic disease,* it is respectfully submitted that claims 12 and 47 are enabled and now meet the requirements of 35 U.S.C. §112, first paragraph.

Regarding the Examiner's rejection of claims 1, 2 and 6-16 (section 7, page 6 of the office action), it is respectfully submitted that with the amendment to the claims, practicing the invention as claimed is clearly enabled. Note that the ITA measured is that which binds to monoclonal antibody B152, the binding of which is specific for ITA and O-linked glycosyl residues (see the enclosed Birken article). The measurement of intact hCG and beta hCG is well known in the art. The measurement of ITA using monoclonal antibody B152 is enabled. It is therefore respectfully submitted that the measurement of hCG, beta hCG and ITA as claimed in the biological sample is enabled, inasmuch as measurement of hCG, beta hCG and ITA in urine, plasma or serum is relatively facile and diagnosing the conditions as claimed flows directly and readily from those measurements. Inasmuch as the measurement of each of hCG, beta hCG and ITA as claimed is enabled, practicing the remaining steps of the claimed invention to determine the existence of the condition is also enabled and facile. The method now clearly reflects the fact that the condition is detecting the presence or absence of invasive trophoblast cells in a patient, not the sample.

Regarding the argument that the claimed method is not enabled, Applicant notes that the amount of intact hCG and optionally, beta hCG as claimed may be measured using any number of methods which are available in the art and are well described in the

literature. In addition, as noted, commercial immunoassays may also be utilized to measure hCG and beta hCG and may be preferably used. These may be used directly or adapted with minor variation in order to obtain an amount of hCG in a sample. Antibodies are readily available commercially which may measure intact hCG and ITA, and optionally, beta hCG. Regarding the measurement of ITA, the preferred method for measuring ITA in a sample is through the use of monoclonal B152, which is readily available and is claimed. Thus, all of the components for practicing the invention are available and well known in the art, all of the steps are well known and practicing the method which simply relies on well known steps already known in the art using components which are readily available in the art evidences that the claimed method is clearly enabled. It is thus respectfully submitted that the presently pending claims are enabled.

Turning to the Examiner's rejection of the previously pending claims 1-16 that those claims fail to meet the requirements of 35 U.S.C. §112, first paragraph for failing to provide an adequate written description, Applicants respectfully traverse the Examiner's rejection. The present claims now adequately directed to measuring the amount hCG (intact hCG plus ITA alone or in combination with beta hCG) which is measured to provide the presently claimed method. That is now adequately described in the specification and set forth in the claims. A review of the claimed subject matter and the specification clearly evidences that the present invention is now in compliance with the requirements of 35 U.S.C. §112, first paragraph as related to the written description requirement. The present invention must be seen to be in compliance with the requirements of 35 U.S.C. §112, first paragraph.

The Rejection of Claims 1, 2, 5-16, 46 and 47 under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 1, 2, 5-16, 46 and 47 under 35 U.S.C. §112, First Paragraph as not containing a written description of the invention. Applicant respectfully submits that the instant claims comply with the requirement for written description. Noting that the Examiner has indicated that he accepts that there is support

in the specification for the use of the term "gestational trophoblastic disease", Applicant respectfully submits that the instant claims are now in compliance with the requirement of 35 U.S.C. §112, First Paragraph. Applicant respectfully requests the Examiner to withdraw his rejection on these grounds.

The Rejection/Objection of Claim 12

The Examiner has objected to claim 12 because the word "determining" was misspelled. Appropriate correction has been made, thus obviating the Examiner's objection to claim 12.

The Rejection of Claims 1, 2, 6-8, 10 and 11 under 35 U.S.C.§102(b)

The Examiner has rejected claims 1, 2, 6-8, 10 and 11 under 35 U.S.C. §102(b) as being anticipated by Kobata, *Biochemie,* 1988, 70: 1575-1585 ("Kobata"). It is the Examiner's continued contention that Kobata teaches a method of measuring ITA in a urine sample and on the basis of the percentage of ITA compared to the total amount of hCG in the sample, detecting invasive trophoblast cells if the percentage of ITA in the sample is greater than 30% of the total amount hCG. Applicant respectfully traverses the Examiner's rejection. It is noted that the variant of hCG which is measured by Kobata is *not* the same as the variant measured by present invention. Indeed, it is the use of monoclonal antibody B152, which is specific for ITA according to the present invention, and the O-glycosyl residues of ITA which clearly distinguishes the present invention over the teachings of Kobata.

Essentially Applicant's method is clearly patentable and *not* anticipated by the method of Kobata simply because Kobata teaches measuring a variant of hCG which is not ITA, as that term has been defined in the specification. In particular, Kobata is directed to measuring an N-linked glycosylated variant of hCG, not a glycosylated version of hCG containing O-linked glycosyl as in the present invention (note that ITA, unlike the Kobata glycosylated hCG, contains both N-linked and O-linked glycosyl

groups, whereas Kobata contains exclusively N-linked groups). This is explained in great detail in the previously submitted Valmu, et al. article and is further emphasized in the enclosed Birken article. Moreover, the Birken article clearly evidences that the use of monoclonal antibody B152 is specific for O-glycosyl linkages of ITA, not the N-linked sugars which are measured by Kobata. Inasmuch as there are a number of glycosylated variants, it is the type of variant which will determine the accuracy of the assay and whether or not invasive trophoblast cells exist in a patient. In the case of Kobata, Kobata is measuring N-linked glycosylated versions of hCG, not ITA of the present invention, which is a hyperglycosylated variant of hCG which contains both N-linked and O-linked glycosyl groups and which is specifically measured by monoclonal antibody B152, which is selective and specific for the O-glycosyl residues, and does *not bind to* N-glycosyl residues as is taught by Kobata. The present method is clearly distinguishable over Kobata and novel.

Note that the definition of ITA, the O-linked glycosylated variant which is measured in the present invention, is set forth in the specification at page 5, in the second full paragraph. This is the variant which Applicant has focused on and to which the present invention is directed to measuring. This is ***not*** what Kobata is measuring. The two types of hyperglycosylated variants of hCG, i.e., those of Kobata and those of the present invention, are quite distinguishable. This is clearly presented in Valmu, et al. at page 1213 and in particular, in the first full paragraph bridging the first and second columns. That disclosure clearly evidences that Kobata is measuring an N-glycosylated variant of hCG, not the variant ITA of the present invention. Moreover, Valmu, et al. points out that the Kobata N-glycal hCG variant could not be detected by the mass-matching approach because the structures carry the same mass as ordinary biantennary N-glycals. This structural feature of the hCG variant measured by Kobata makes its measurement difficult given its similarity in structure to related variants. This would also explain the relatively low accuracy of the Kobata method compared to the method of present application. It is clear from Valmu, et al. that the Kobata measured hCG variants are clearly distinguishable from ITA, the variant measured in the present invention. See Valmu, et al. also at page 1216, first full paragraph in the left column. Given the fact that

the present invention and Kobata measure two distinguishable types of glycosylated hCG as evidenced by the teachings of Valmu, et al. and the teachings of Birken (enclosed), the method of Kobata does not anticipate the present invention.

In contrast to the present method, Kobata *only* deals with and measures N-linked glycosylated hCG, not the O-linked glycosylated hCG which is measured in the present invention. Thus, the present invention clearly distinguishes over Kobata in measuring a different hCG variant (known as HhCG or ITA) than Kobata. Thus, because Kobata is not directed to the same or identical method as the present invention (because of the clearly distinguishable variants which are measured in the disclosure of Kobata vs. the present method), Kobata does not and *cannot* anticipate the present invention.

Valmu, et al. clearly shows that the O-linked glycosylated hCG variant ITA, which is measured in the present method, is distinguishable from the N-linked glycosylated hCG which is measured by Kobata, as discussed above and Birken clearly teaches that monoclonal antibody B152 is specific for O-linked glycosyl residues of ITA of the present invention. Not only does Valmu, et al. show the distinction between the N-linked and O-linked glycosylated variants of hCG, but also points to the superiority of measuring ITA- which is the only significant and consistent change in choriocarcinoma. Birken clearly teaches the specificity of measuring ITA as defined in the present application. Thus, Kobata, clearly is directed to measuring a different hCG variant and the disclosed method clearly does not anticipate the present invention.

The Examiner continues to argue that Applicant, in order to distinguish Kobata, recites limitations which are not in the claims. Applicants respectfully traverse the Examiner's arguments. The fact that Applicant recites ***ITA*** in the claims ***is*** *a distinguishable limitation in the claims* because ITA, which contains O-linked glycosyl groups as defined in the specification and as described in Valmu, et al., is distinguishable from the hCG variant measured in Kobata, which is directed to a different form of glycosylated hCG containing N-linked glycosyl groups. These hCG variants are clearly not the same and are clearly distinguishable. Given the fact that ITA and the hCG variant

of Kobata are distinguishable, as evidenced by the clear description in Valmu, et al., and given the fact that Birken clearly teaches that monoclonal antibody B152 is specific for O-linked glycosyl ITA, not the hCG variant of Kobata, the present claims are not anticipated by the art of record and the distinguishable limitation *is found* in the presently pending claims.

The Rejection of Claims 5 and 46 under 35 U.S.C.§103(a)

The Examiner has rejected claims 5 and 46 as being obvious over Kobata, as evidenced by Cole and Butler, *J. Reproductive Medicine,* June, 2002, 47: 433-444 ("Cole and Butler") as applied to claims 1, 2, 6-8 10 and 11 ad further in view of Cole, et al., *Clin. Chem.*, 1997, December 2001 47: 308-315 ("Cole") for the reasons which are stated in the office action at section 11, pages 15-16. The Examiner contends that it would have been obvious to one of ordinary skill in the art at the time of the invention to provide the invention of claims 5 and 46 given the teachings of Kobata and the teachings of Cole. Applicant respectfully traverses the Examiner's rejection.

The invention of claims 5 and 46, which are both dependent on claim 1, are directed to a method for measuring the total amount of intact hCG and ITA in a biological sample from a patient at risk for gestational trophoblastic disease or quiescent gestational trophoblastic disease as claimed and determining the amount of total intact hCG plus ITA with the amount of ITA measured *using an immunoassay which determines the selective binding of monoclonal B152 to ITA* in the sample such that a diagnosis of gestational trophoblastic disease or quiescent gestational trophoblastic disease may be made. It is respectfully submitted that the present invention is non-obvious over the teachings of Kobata, as evidenced by Cole and Butler and further in view of Cole.

The teachings of Kobata are described in great detail hereinabove. It is axiomatic that Kobata, which teaches the measurement of variants of hCG which contain N-linked glycosyl residues *which are not even detected by monoclonal antibody B132*, does not

teach or suggest the present invention. Those clearly inadequate teachings of Kobata are not somehow obviated by Butler and Cole and/or Cole, each of which fails to disclose or suggest ITA measurement with monoclonal antibody B132 as being critical for determining whether or not a patient is at risk for gestational trophoblastic disease or quiescent gestational trophoblastic disease. Indeed, if anything, the teachings of Kobata arguabley *teach away* from the present invention inasmuch as Kobata teaches the measurement of a different hCG variant, *not* the variant which is used to diagnose gestational trophoblastic disease or quiescent gestational trophoblastic disease according to the present invention. Given that there is no teaching or suggestion in any of Kobata, Cole and Butler, or Cole to measure ITA as defined in the present specification (by measuring O-linked glycosylation through the specific binding using monoclonal antibody B132), it is respectfully submitted that the present invention is non-obvious over the teachings of the cited art.

For the above reasons, Applicant respectfully asserts that the claims set forth in the amendment to the application of the present invention are now in compliance with 35 U.S.C. Applicants respectfully submit that the present application is now in condition for allowance and such action is earnestly solicited.

Applicants have neither canceled nor added any claim. No fee is therefore due for the presentation of this amendment. A notice of appeal is being send under separate cover. The Commissioner is authorized to charge the $255 fee for the appeal to deposit account 04-0838. If any additional fee is due or any overpayment has been made, please charge/credit Deposit Account No. 04-0838.

Should the Examiner wish to discuss the present application in an effort to advance its prosecution, the undersigned attorney may be reached at the telephone number set forth hereinbelow.

Respectfully submitted,

COLEMAN SUDOL SAPONE, P.C.

By: _____
Henry D. Coleman
Reg. No. 32,559

Dated: August 27, 2008
Enclosure (Birken Reference)

714 Colorado Avenue
Bridgeport, CT 06605-1601
203-366-3560

Certificate of Facsimile Transmission

I hereby certify that this correspondence is being sent by facsimile transmission to the Examiner Peter J. Reddig, in Group Art Unit 1642 of the United States Patent Office in Alexandria, VA 22313-1450 on August 27, 2008.

_____
Henry D. Coleman (Reg. No. 32,559)

Mini Review

TumorBiology

Tumor Biol 2005;26:131–141
DOI: 10.1159/000086484

Received: November 13, 2004
Accepted after revision: January 26, 2005
Published online: June 20, 2005

Specific Measurement of O-Linked Core 2 Sugar-Containing Isoforms of Hyperglycosylated Human Chorionic Gonadotropin by Antibody B152

Steven Birken

Department of Obstetrics and Gynecology, Columbia College of Physicians and Surgeons, New York, N.Y., USA

Key Words
Glycoprotein hormones · Hyperglycosylated human chorionic gonadotropin · Core 2 human chorionic gonadotropin isoforms · Antibody B152

Abstract
There have been a significant number of reports on the clinical utility of measurement of 'hyperglycosylated' isoforms of the pregnancy hormone, human chorionic gonadotropin (hCG). Although there are a variety of hCG isoforms which can be termed 'hyperglycosylated', the measurements were all made using a unique antibody designated B152. This antibody was raised using a choriocarcinoma-derived form of hCG, which was hyperglycosylated with N- and O-glycans and was also 100% 'nicked' hCG. Antibody B152 was recently mapped to a linear epitope around a single O-glycan on the β-subunit of hCG at residue number 132. Thus, the antibody can only measure isoforms of hCG that possess a core 2 type of branched O-glycan on this portion of the hCG β-subunit. Isoforms that are hyperglycosylated in the hCG α-subunit or only on the N-glycans of hCGβ will not be recognized by antibody B152. Apparently, measurements of these core 2 hCG isoforms have important clinical application in early pregnancy during which they are the predominant isoform of hCG until the 6th week of gestation. The secretory pattern of these isoforms can be used to predict the health status of the pregnancy in fertility clinics. Moreover, the measurements of these core 2 hCG isoforms are more useful than standard hCG for the prediction of Down syndrome pregnancies. The core 2 isoforms are also of important use in cancer diagnosis and monitoring since their concentration appears to correlate with malignancy.

Background

During the past 5 years, there has been a series of reports on the potential clinical importance of measurement of certain isoforms of human chorionic gonadotropin (hCG) that have been termed 'hyperglycosylated hCG' or 'invasive trophoblastic antigen' (ITA) or 'early pregnancy isoform of hCG' [1–6]. Measurements of these variant forms of hCG have applications in prenatal diagnosis, specifically Down syndrome [1–3, 6–12], in assessment of the health and likely success of early pregnancy in the fertility clinic [4, 13], and for both diagnosis and management of hCG-secreting cancers [14–16]. The purpose of this review is to define what is known about the hCG isoforms being measured, to detail the characteristics of the antibody which is used to define these hCG isoforms and to describe the variety of potential clinical applications and complications in making these measurements. The utility of new protein markers in tumor diagnostics has been reviewed by important workshops re-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

Accessible online at:
www.karger.com/tbi

Steven Birken
Department of Obstetrics and Gynecology
Columbia College of Physicians and Surgeons, 630 W 168th Street
New York, NY 10032 (USA)
Tel. +1 212 305 5755, Fax +1 212 305 1599, E-Mail sb18@columbia.edu

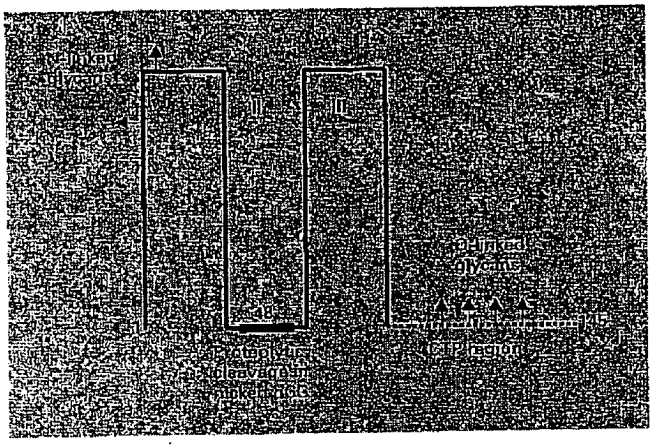

Fig. 1. Diagram showing the structure of the β-subunit of hCG. Each hCG subunit is composed of three loops [51, 52]. HCGβ contains two large N-glycans within loop I and four small O-glycans within the COOH-terminal region (dashed line), which is a unique peptide addition not present in the highly homologous hLH β-subunit. The middle of loop II contains a protease-sensitive area around residue 48 which is cleaved in the hCG produced by many cancer patients resulting in hCGn and free subunit hCGβn.

ported earlier in this journal [17, 18]. While hCG and its subunits have long been recognized as tumor markers or even tumor growth factors [17, 19–22], the hyperglycosylated hCG isoforms described in this review appear to be markers of both normal as well as abnormal clinical states.

Development of Antibody B152 Which Defines These Isoforms

The isoforms of hCG bind better to antibody B152 than the standard WHO form of hCG (mid-pregnancy urinary hCG) and have been variously termed 'ITA', 'hyperglycosylated hCG' or 'early pregnancy forms of hCG' [3, 4, 13, 23, 24]. It should be understood that there is more than one isoform that meets the definition of binding to antibody B152. In order to appreciate what antibody B152 measures, one must review its origin and characterization.

About a decade ago, we collaborated with the laboratory of Dr. Laurence Cole in an attempt to produce antibodies to 'nicked hCG' (hCGn) which is an isoform of hCG commonly observed in the urine and sometimes in the blood of patients with hCG-secreting tumors and other disorders [25–30]. hCGn is a proteolytically damaged form of hCG which has peptide bond cleavages in its β-subunit between residues 43 and 49 [23, 25, 27, 29–31]. Figure 1 shows linear drawings of hCGβ which is the immunological focus of this review. hCG consists of the common glycoprotein hormone α-subunit and the hCG-defining β-subunit. Both subunits have a three-loop structure. The hCGβ subunit is found as part of hCG (noncovalently bound to hCGα) as well as in free subunit form in patient specimens. hCGn is hCG whose β-subunit (hCGβn) contains bond cleavages within loop II of hCG, which is shown in figure 1, with residue 48 highlighted as the main region of cleavage in hCGβn. hCGβn combined with hCGα is hCGn commonly found in cancer patients with hCG-secreting tumors. The protease-sensitive area of loop II hCGβ (fig. 1) is thought to be exposed to solvent [25, 27, 32]. Likewise, the βCOOH-terminal region of hCG – C-terminal peptide (CTP) (fig. 1) – is also a region of hCGβ exposed to solvent and easily cleaved free of the remainder of the β-subunit. The CTP portion exists only on hCGβ and does not exist on the highly homologous human luteinizing hormone (hLH). Since this hCG peptide is absent from hLH, it has been a focus of much immunologic study as an immunogen unique to hCG without the possibility of producing an antibody which has crossreaction to hLH, resulting in generation of assays specific to hCG in the presence of hLH [33–35]. hCGβcf (which is a degradation product consisting of half of the hCGβ molecule, namely disulfide-linked residues 6–40 coupled by disulfide bridges to 55–92) is the main urinary metabolite of hCG and found at high concentrations in the urine of pregnant women as well as the urine of cancer patients with hCG-secreting malignancies [32, 36]. This hCGβ metabolite is not recognized at all by antibody B152 and is therefore not a subject of this review.

The purpose of the attempt to produce monoclonal antibodies to hCGn was to develop immunoassays to analyze the expression of this form of hCG in the blood and urine of cancer patients. Monoclonal antibodies were developed at Columbia using an hCG isoform isolated from a patient with choriocarcinoma in Larry Cole's laboratory at Yale University [23, 37]. This hCG isoform, named preparation C5, was unusual, even for a cancer patient, as in addition to hyperglycosylated (tri- and tetra-antennary) N-glycans, its O-glycan structures were 100% core 2 (fig. 2) in structure rather than mostly core 1 glycans as is the case for the WHO hCG standard made from mid-pregnancy urine [37]. Figure 1 shows that hCGβ has both two N-linked glycans and four O-linked glycans, the latter all located on the CTP portion of hCGβ. Figure 2 shows the nature of the difference between the linear core

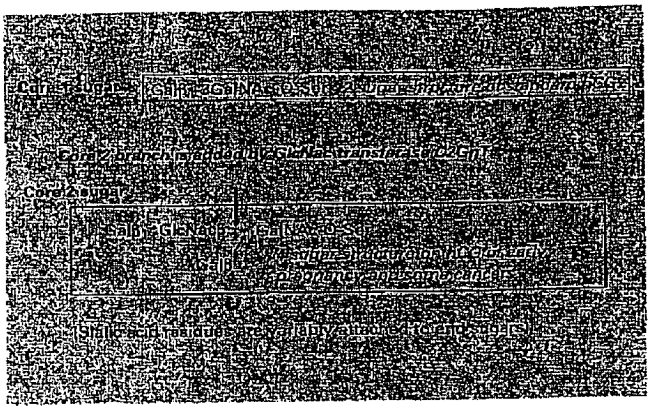

Fig. 2. Comparison of the structures of core 1 sugar core present in standard mid-pregnancy hCG and core 2 sugar core present in early pregnancy hCG and some cancers which produce hCG. The core 2 sugar contains a branch introduced by the GlcNac transferase C2GnT as shown. The core 2 sugar structure is part of the specific epitope site recognized by antibody B152. This figure was modified from Birken et al. [43] with permission of Elsevier, © 2003.

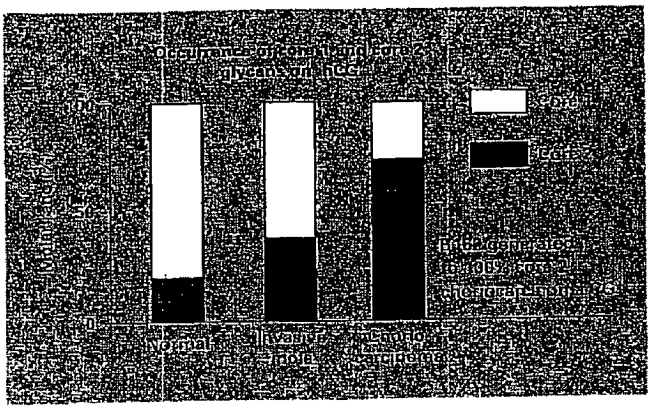

Fig. 3. Comparisons of the relative molar ratios of the presence of core 1 compared with core 2 sugars as determined by chemical analysis of pools of hCG extracted from normal mid-pregnancy urine (normal), urine from a woman with an invasive hydatidiform mole pregnancy (invasive mole) and urine from a woman with choriocarcinoma (choriocarcinoma). This study by Amano et al. [41] indicated the association of core 2 biosynthesis with malignancy, and this trend agrees with measurements made by antibody B152 which is specific to hCG which contains core 2 O-glycan.

1 O-glycan structure and the branched core 2 O-glycan structure which was elaborated by the activity of the transferase C2GnT [38–40]. It was known a number of years earlier from the chemical analyses of Kobata's laboratory that mid-pregnancy hCGβ was about 90% core 1 and 10% core 2, while invasive mole hCG and choriocarcinoma hCG were much higher in core 2 structure [41], as shown in figure 3. The choriocarcinoma hCG C5 was 100% hCGn and also fortuitously contained essentially 100% of the core 2 structure [37, 39, 40].

Two monoclonal antibodies were selected on the basis of high affinity to the choriocarcinoma hCGn C5, B151 and B152. It was found that while antibody B151 did indeed preferentially bind to hCGn, both choriocarcinoma hCGn and pregnancy hCGn, B152 preferentially bound to choriocarcinoma hCGn but much less to pregnancy hCGn [4, 23]. These characterization experiments are illustrated in figure 4. Since the main difference between the choriocarcinoma hCGn immunogen C5 which is 100% nicked and hCGn of mid-pregnancy urine which is also 100% nicked is carbohydrate differences, it was clear that B151 was directed towards the new epitope that appeared upon nicking or cleavage of hCGβ in the 43–49 region, while B152 was oblivious towards this epitope but recognized an as yet undefined carbohydrate difference between the two isoforms of hCG.

Antibody competition experiments soon indicated that hCG molecules captured by B152 could not be simultaneously bound by detection antibodies directed to the CTP region of hCGβ as well. This indicated that its epitope was likely close to or within the CTP β-region. Based on studies over the past two decades, the CTP region of hCG is thought to be quite exposed to solvent, to have a mobile structure and is known to usually induce linear rather than conformational antibodies [33, 35]. This result was found whether synthetic peptide analogs or natural CTP conjugated to carrier proteins were employed as immunogens [33, 42]. Consequently, a simple dot blot experiment was performed to test whether B152 was directed to a conformational or a linear epitope by spotting hCGβ and reduced, denatured hCGβ as well as similarly treated C5 choriocarcinoma hCG on a polyvinylidene fluoride blotting membrane; then, each of the spots was reacted with either known conformationally directed hCGβ antibodies or linearly directed hCG antibodies [13]. This experiment is shown in figure 5. Antibodies B201 and B207 are both directed to conformational-dependent epitopes in hCGβ which is clearly shown in the dot blots in which only the native forms of both hCG isoforms hCGβ and C5 are darkly stained, while the reduced forms are very faint. In contrast, the opposite is observed when staining with a linearly direct-

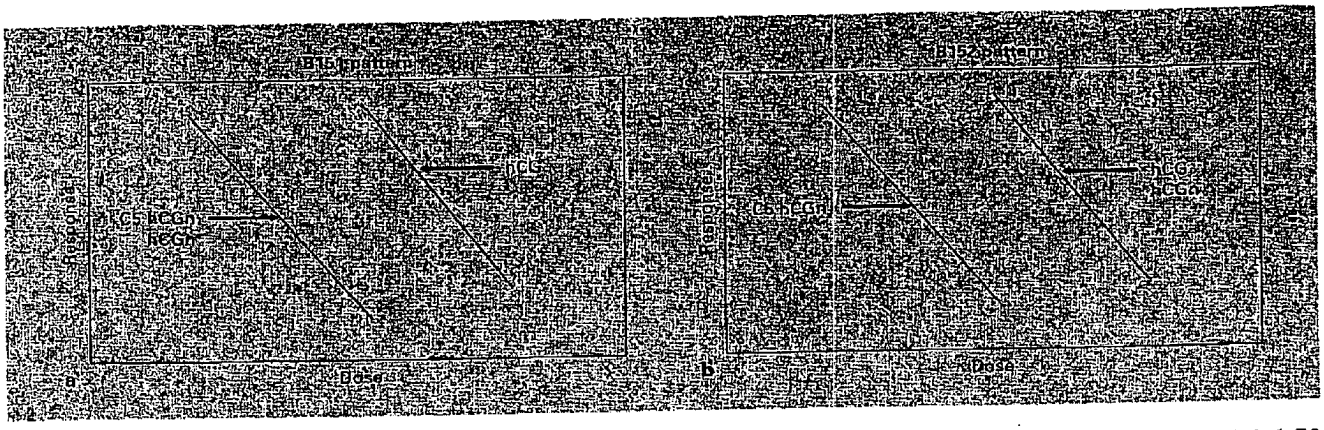

Fig. 4. **a** Dose-response curves of antibody B151 in a liquid-phase radioimmunoassay using radiolabeled C5 choriocarcinoma hCGn which is 100% hCGn and hyperglycosylated on both N- and O-glycans and unlabeled competitors: C5 choriocarcinoma hCG, hCGn (WHO standard 99/642) [46, 47] and standard hCG (WHO 99/688) [4, 47]. This study indicates that B151 can differentiate forms of hCGn regardless of their carbohydrate differences. hCGn is standard in carbohydrate content while C5 choriocarcinoma hCGn is termed 'hyperglycosylated'. **b** Dose-response curves of antibody B152 in a liquid-phase radioimmunoassay using radiolabeled C5 choriocarcinoma hCGn which is 100% hCGn and unlabeled competitors: C5 choriocarcinoma hCG, hCGn (WHO standard 99/642) and standard hCG (WHO 99/688) [46, 47]. Since standard hCG and standard hCGn both compete poorly and only choriocarcinoma hCGn competes well, this study implies that B152 is directed to carbohydrate differences (choriocarcinoma hCG is hyperglycosylated) rather than to differences based on nicking of hCG.

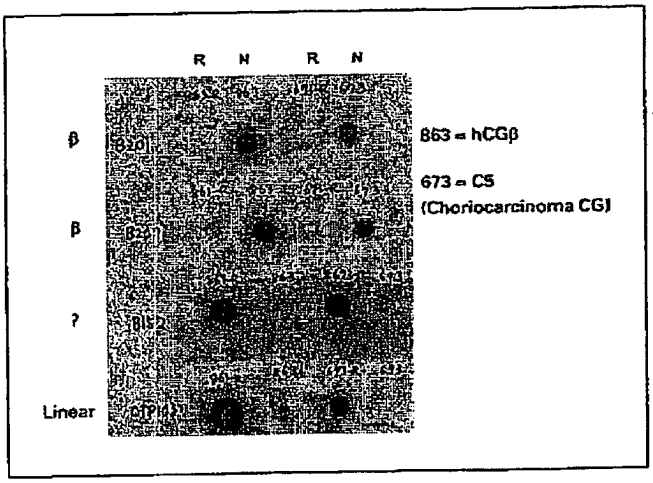

Fig. 5. Dot blot study of staining of standard hCGβ and of choriocarcinoma hCGn with four monoclonal antibodies. Antibodies B201 and B207 were known to be conformationally directed, while antibody CTP103 was made to synthetic peptide and is likely to be a linearly directed antibody. These were compared with antibody B152 (?) to ascertain if it was conformationally or linearly directed. The dot-blotted proteins were hCGβ (lot 863 which is identical to WHO 99/650) [46, 47] and C5 choriocarcinoma hCGn which were both spotted in reduced (R) or native (N) states (boiled in SDS buffer with or without reductant). It is clear that conformationally directed antibodies mainly or solely stain native hCG forms while the linearly directed antibody (CTP103) mainly stains the reduced form. From this study, B152 appears to be a linearly directed antibody since it prefers to stain reduced hCG forms very similar to CTP103. This illustration is a modification of a figure published by Kovalevskaya et al. [13], © Society for Endocrinology (2002). Modified with permission.

ed CTP antibody (CTP103), made to a synthetic peptide analog of the CTP, which stains the reduced form well but not the native molecules. B152 reacts similarly to CTP103; thus, one can conclude that B152 is likely directed to a linear portion of the molecule.

Figure 6 shows liquid-phase dose-response curves for antibody B152 using radiolabeled C5 choriocarcinoma hCG immunogen and a variety of unlabeled competitor forms of hCG and hCGβ [23]. As expected, C5 hCGn and C5 hCGnβ compete well, as the immunogen for the antibody. The group of curves on the right side of the graph are all derived from mid-pregnancy urine hCG, and all contain only about 10% of the high sugar forms recognized by B152. However, it is clear that even free CTP is active in this study, as well as completely reduced and alkylated hCGβ and desialylated forms of hCGβ [23].

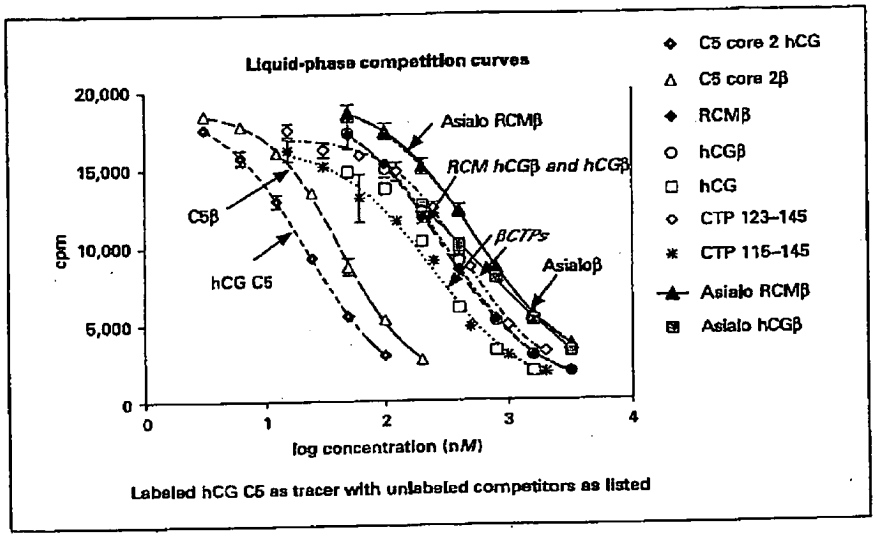

Fig. 6. Liquid-phase dose-response curves for antibody B152 and radiolabeled C5 choriocarcinoma hCGn. Unlabeled competitors were C5 choriocarcinoma hCGn and C5 hCGβn, both of which contain 100% core 2 O-glycans specifically recognized by antibody B152. All other competitors were derived from standard hCG which contains 10–15% core 2 O-glycans with the majority of molecules containing core 1 O-glycans: reduced carboxymethylated-β (RCMβ), hCGβ, hCG, COOH-terminal hCG natural peptides of residues 123–145 and 115–145 (CTP), desialylated RCMβ (asialo, RCM hCGβ) and desialylated β (asialo, hCGβ). All standard materials were much less potent in this assay since they contain only 10–15% core 2 type O-glycans compared with C5 choriocarcinoma hCGn molecules which are 100% core 2 in structure. This study indicates that the main B152 epitopes are contained in the free COOH peptides and that reduction of the molecules or desialylation makes little difference in antibody recognition.

Later, more detailed epitope mapping studies performed in collaboration with Dr. William Moyle who constructed a series of recombinant hCG analogs with single deletions of each of the O-glycan groups (by substituting Ala for Ser) permitted mapping of the main glycan-binding regions of B152 to β-serine 132 [43] (fig. 7). When the O-glycan at serine 132 is not present, B152 fails to bind to hCG, whereas removal of the other glycan sites have no effect on binding. A monoclonal antibody developed earlier using natural mid-pregnancy hCG CTP conjugated to a carrier protein, CTP104, proved to be directed more distally to the last O-glycan region (fig. 7) [43]. Of course, both antibodies are specific to hCG and thus recognize part of the CTP chain along with the glycan. Neither antibody exhibits significant background binding to either urine or blood matrix.

B152 is remarkably specific in terms of its requirement for the core 2 type O-glycan structure of the hCG isoform. This was found by an experiment shown in figure 8 which involved the use of immobilized B152 and immobilized CTP104, both attached to a matrix of Biorad Affi-Gel 10. In this experiment, new immobilized B152 and CTP104

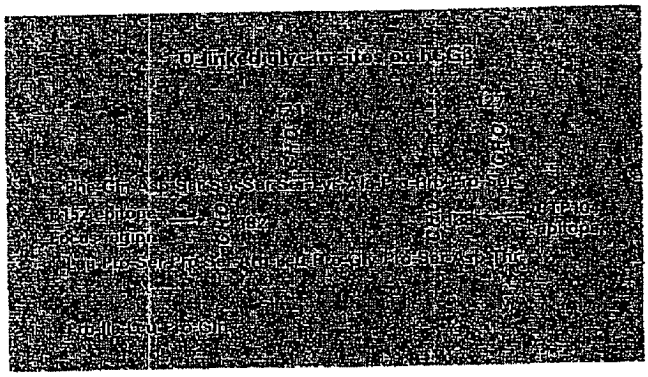

Fig. 7. Approximate location of the epitopes of antibodies B152 and CTP104 on the CTP portion of hCGβ. B152 binds in the area of the O-glycan on residue 132 of the β-subunit. It also requires that this glycan be of the core 2 type of sugar structure (fig. 2). CTP104 binds around O-glycan on residue 138 but will bind to either core 1 or core 2 O-glycan structures. β-residue numbers are indicated. This figure is reproduced from Birken et al. [43] with permission of Elsevier, © 2003.

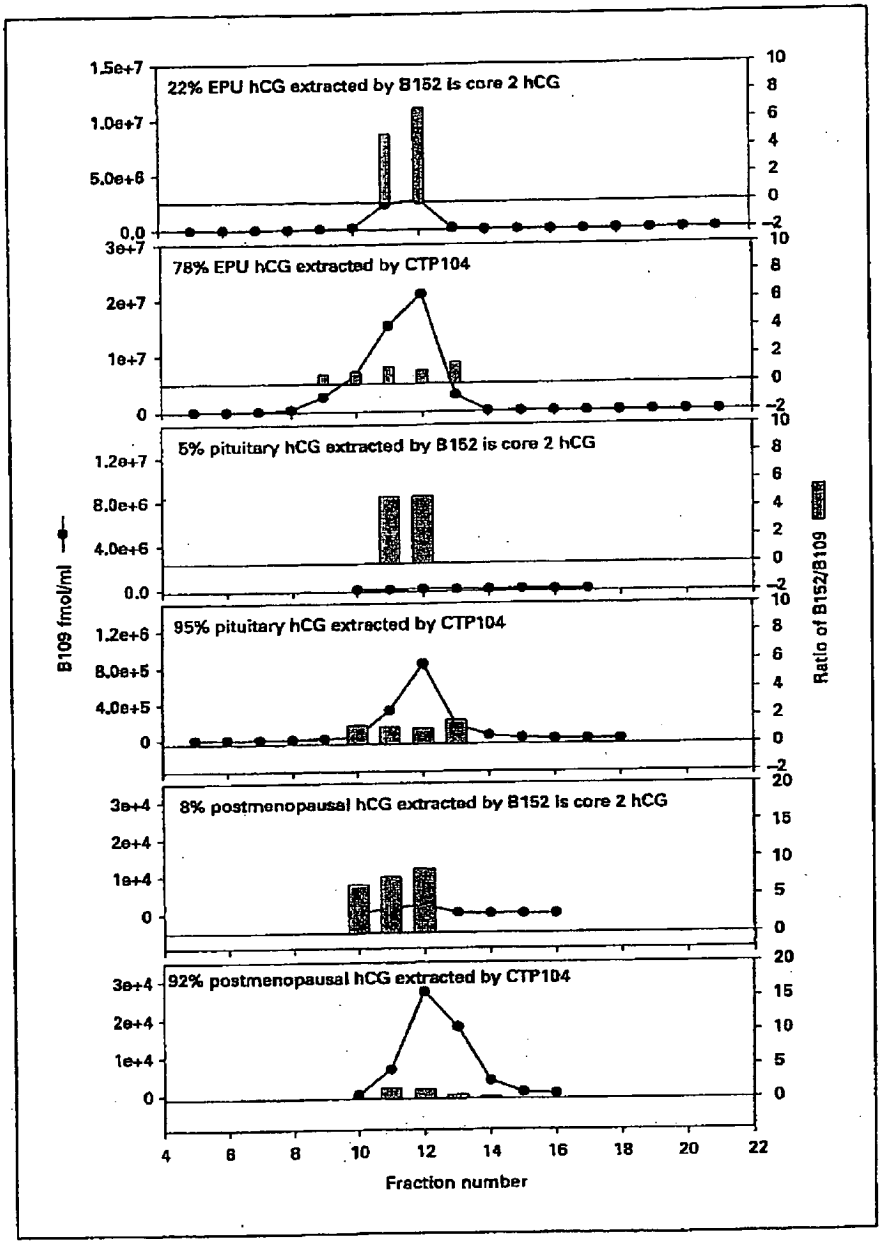

Fig. 8. Immunoaffinity study comparing hCG isoform extraction differences by immobilized CTP104 and B152 columns. Each extract indicated in the figure – early pregnancy urine (EPU), human pituitary aqueous extract and postmenopausal urine concentrate – was divided into two equal portions, loaded and then eluted from each column (x-axis shows fraction numbers as eluted from the gel filtration columns). Next, the eluants were gel filtered and each fraction was assayed by both the B109-B207* and the B152-B207* assays. The areas under the curves for total hCG (B109-B207*) and the relative amount of hCG extracted by either B152 or CTP104 were calculated as a percentile by calculating the total areas under the curves for both extractions as equal to 100%. The ratios of B152/B109 indicated whether the hCG predominantly was the hyperglycosylated B152 core 2 isoform of hCG. It was found that the particular first trimester pregnancy urine pool used in this study appeared to contain 22% core 2 hCG, while pituitary extracts and postmenopausal urine hCG (also pituitary) contained only 5–8% of this form. CTP104 extracted a mixture of both core 1 and core 2 hCG isoforms. This figure is modified from Birken et al. [43], with permission of Elsevier, © 2003.

gels (never used before) were divided into six portions. Likewise, three starting materials (an early pregnancy urine pool, an aqueous human pituitary extract pool and a postmenopausal urine pool) were each precisely divided in two, and each portion was absorbed and then eluted from the both previously unused immunoaffinity matrices. The hCG isoforms were quantified fraction by fraction by two assays: B152, which measured the core 2 type isoforms, and B109, which measured total hCG present. Calculations showed that a pool of early pregnancy urine (weeks 3–8) contained about 22% of the core 2 isoforms and the remainder was standard core 1 hCG [43]. This pool contained a significant quantity of standard hCG isoforms because it extended beyond week 6, as will be

described later. This experiment also showed that pituitary tissue extract contained only 5% of the isoform recognized by B152, and postmenopausal urine contained only 8% of this isoform (the latter two sources should be about the same, since all postmenopausal hCG should derive from the pituitary unless the patient has an hCG-secreting malignancy). While the CTP of pituitary hCG has not yet been characterized in detail [44], the great specificity of B152 is clear from this experiment, since even at very high hCG concentrations, only the specific B152-recognized isoform binds to the antibody. This conclusion is possible since all the hCG eluted from the B152 column displayed a high B152/B109 ratio indicative of the core 2 hCG isoform, while the hCG eluted from the CTP104 column was a mixture of both isoforms. In addition, the pituitary and postmenopausal hCG was only poorly bound by B152 despite its high concentration, while CTP104 essentially bound all the pituitary and postmenopausal urinary hCG in the sample.

Forms of 'Hyperglycosylated' hCG Recognized by Antibody B152

All of the reports on immunological measurements of hyperglycosylated hCG during the past several years have employed antibody B152, since this is the only antibody reported to detect such molecules. One must realize in this case that the antibody is defining the isoforms being termed 'hyperglycosylated', and this definition is very narrow due to the specificity of the antibody. Since B152 recognizes a very small portion of the hCG molecule, namely the region around β-residue 132, we considered the other isoforms present and which would or would not be measured by B152. Some possible structures are shown in figure 9. Two forms are shown that have standard core 1 O-glycans at residue 132 (isoforms A and B). These would not be measured by B152. However, an isoform with extra carbohydrate at its N-groups is clearly a form of hyperglycosylated hCG but would not be included as a B152-recognized hyperglycosylated hCG isoform (fig. 9, isoform B). Likewise, the 3rd isoform in figure 9 (isoform C) would bind to B152 but may have standard biantennary N-glycan groups and does not resemble the C5 immunogen which is fully hyperglycosylated on both N- and O-glycan groups. Therefore, the reference to measurement of 'hyperglycosylated hCG' or 'ITA' or other similar names that imply a single type of isoform is misleading. Until the various isoforms of hCG are isolated and characterized from both pregnancy as well as cancer

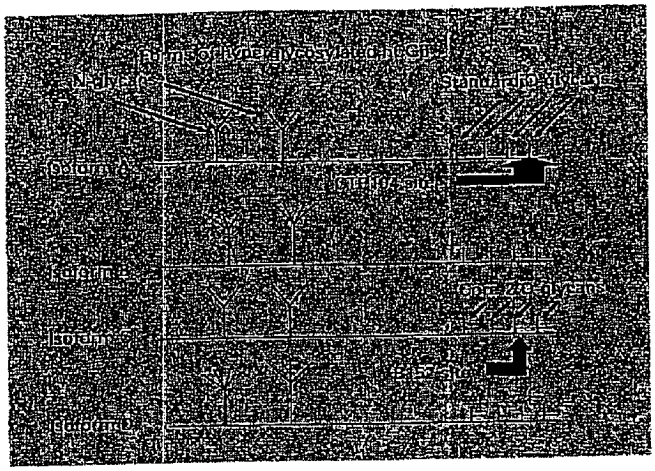

Fig. 9. Comparison of some of the various possible isoforms of the β-subunit of hCG which would form part of hyperglycosylated hCG and indicates the very small region of the molecule that forms the epitopes of antibodies B152 and CTP104. Only changes in these epitope regions would effect recognition of each isoform by these antibodies directed to small, linear regions of hCGβ. Isoform A contains biantennary N-glycans and core 1 O-glycans and is the predominant hCGβ isoform of standard mid-pregnancy hCG. Isoform B has hyperglycosylated tri- and tetra-antennary N-glycans but standard core 1 O-glycans. Isoforms A and B are not recognized by antibody B152 but would be measured by antibody CTP104 which recognizes all the isoforms shown in this figure. Hyperglycosylated hCG with isoform B structure would not be recognized by antibody B152 as a hyperglycosylated isoform. Isoforms C and D are recognized by antibody B152. Isoform C with standard biantennary N-glycans but hyperglycosylated core 2 O-glycans would be recognized by antibody B152 as a hyperglycosylated hCG and may be one type existent in early pregnancy. Isoform D is the immunogen used to produced B152 and was derived from an unusual choriocarcinoma hCGn which was both 100% nicked and fully hyperglycosylated at both N- and O-glycans, meaning N-glycans were tri- or tetra-antennary and O-glycans were core 2 structures.

patients and the reactivity of each isoform with B152 is defined, it may be best to refer to isoforms measured by B152 simply as B152-recognized hCG isoforms or core 2 containing hCG isoforms.

Measuring Core 2 hCG Isoforms

Currently, there is no accepted standard for use of antibody B152. Part of the reason is apparent in figure 9. B152 can measure any hCG isoform which contains a core 2 glycan structure on β-residue 132. What then is an appropriate standard? Clearly, the immunogen itself

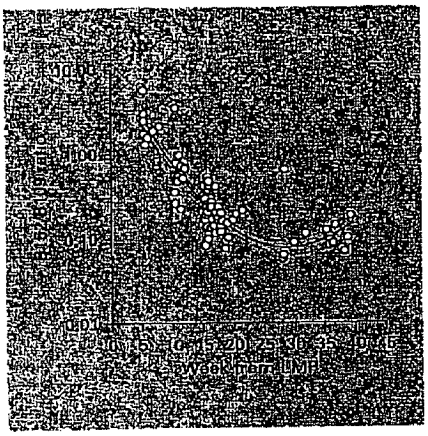

Fig. 10. Measurement of B152-recognized forms of hCG during early pregnancy by use of the ratio measurement technique of dividing molarity of B152 measurement by those of B109 total hCG measurements when both assays are read off the same WHO standard hCG curve. High ratios indicate most or all of the hCG which is the core 2 type recognized by B152. This form declines dramatically starting at about 6 weeks of pregnancy and is very low in the second and third trimester. This figure is reproduced from Kovalevskaya et al. [4], © Society of Endocrinology (1999). Reproduced with permission.

which was isolated from a choriocarcinoma patient is not an appropriate standard since it has peptide bond cleavages within loop II (a form of hCGn) and also hyperglycosylated N-glycans, typical of many cancer tumor-produced hCG isoforms. In 1998, O'Connor et al. [45] showed for the first time that hCG produced in early pregnancy contained a high proportion of hCG with strong affinity to B152 (they called it 'early pregnancy' hCG). Early pregnancy hCG may be a better standard for measurement of these isoforms in pregnancy. However, only low concentrations of hCG are produced during early pregnancy and this would be an obstacle in producing an adequate standard. Currently, the media products of various hCG-secreting cell lines are being investigated as potential suitable standard molecule even though these molecules may not be identical in structures to those found in the variety of patient specimens to be measured from healthy pregnancies to diseased pregnancies to cancer patients. Such a tissue culture-produced standard may present the best compromise standard for measurement of isoforms recognized by B152 since it can be produced as a large lot of well-characterized material, and as such, can be proposed as a WHO standard and assigned proper molar units for immunoassay. In Birken et al. [43], it was shown that the current WHO hCG 1st IRP for immunoassay [46, 47] may be used as a standard if a scavenger type approach to measurement is employed. That paper showed that B152 is essentially completely specific for the core 2 hCG isoform of hCG and thus can be used to compete out binding of one of the other βCTP antibodies to determine the fraction of the core 2 isoform present. (Excess B152 was added in doses even up to several micrograms per Elisa plate well containing a sandwich of hCG in which the second antibody was CTP104, which binds both core 1 and core 2 O-glycan forms of hCG. A constant fraction of CTP104 was competed off the hCG no matter how much B152 was added to the well.) Since the CTP antibodies (CTP103 and CTP104) can be used to determine the actual molar quantity of hCG present, the precise molar quantity of the core 2 isoform can be calculated by multiplying the concentration of hCG measured by CTP104 by the fraction of labeled CTP104 competed off the sandwich by the excess B152 added to the well. For example, if the CTP104 assay measures 1 nmol of total hCG present/ml, and 20% of the radiolabeled CTP104 is competed off the bound hCG isoform by excess B152, then 0.2 nmol/ml of the B152 core 2 hCG isoform is present in the specimen [43]. Alternatively, we have been using a semiquantitative ratio approach using the current WHO hCG standard and two measurements, one using B152 and one using a total hCG measurement system. A high ratio semiquantitatively indicated the presence of a high proportion of core 2 or hyperglycosylated hCG isoforms as shown in figure 10, a study on the occurrence of the isoform in early pregnancy [4].

Clinical Applications of Antibody B152

Prediction of the Health of Early Pregnancies

Studies conducted at Columbia utilized the core 2 hCG isoform measurements by the B152 ratio technique in the urine of pregnant women. The WHO hCG standard [46, 47] is used and each sample is measured with both the B152 assay and the general total hCG assay (using antibody B109). Next, the result of B152 measurement is divided by the result for B109 giving a relative proportion of B152 core 2 hCG isoforms present as a pure number. These B152-recognized core 2 hCG isoforms are present at very high levels very early in pregnancy and then decline to the concentrations found in standard mid-pregnancy hCG (i.e. the WHO standard) by weeks 8–10 (fig. 10) [4]. The higher the ratio number, the larger the relative quantity of the core 2 isoform recognized by B152 is

present. Generally, a ratio of 0.5 or greater indicates that a significant quantity of the B152-recognized isoform is present. Ratios as high as 10 were observed in the study shown in figure 10. Furthermore, it was shown that comparisons of these measurements between those women whose pregnancy advanced in a healthy manner compared with those who lost the pregnancy early allowed prediction of which pregnancy was likely to proceed in a healthy fashion [13]. Thus, the B152 assay provides prediction of the normal progress of the pregnancy.

Prediction of Down Syndrome Pregnancy

The laboratories of L. Cole as well as that of Nichols Institute Diagnostics (which produces a commercial measurement system based on B152 on its 'Advantage' automated platform) and Palomaki at the Blood Foundation have done a series of studies showing that measurement of the core 2 hCG isoforms by B152 can provide an improved Down syndrome screening test over that obtained using standard hCG isoform measurements [3, 7–12]. This finding may relate to the correspondence of secretion of these early pregnancy isoforms with the state of differentiation of the placenta. There are some reports that the placenta of Down syndrome pregnancies is less well differentiated compared with healthy pregnancies [48, 49]. In the experiments based on the analysis of media from cultured placental cytotrophoblasts and syncytiotrophoblasts, it has been found that the core 2 hCG isoforms appear to be produced by cytotrophoblasts and not by syncytiotrophoblasts [5]. A combination hCG test which measures both total standard hCG and the core 2 isoforms measured by antibody B152 would be useful in adding additional information to the health of the pregnancy.

Applications to Diagnosis and Monitoring of hCG-Secreting Cancers

Since most hCG-secreting cancers produce significant quantities of the B152-recognized hCG isoforms (choriocarcinoma hCG was the immunogen for the antibody), applications of the assay are important for enhanced and advanced diagnosis and monitoring of therapy for such diseases [24, 50]. In fact, in situations in which women continue to produce very low but abnormal concentrations of hCG long after a pregnancy, especially a pregnancy with a hydatidiform mole, this assay has an important application. It was shown by Cole that a sudden conversion of the secreted hCG to mostly the core 2 isoform was associated with a change to a more malignant condition requiring treatment [50] (fig. 11).

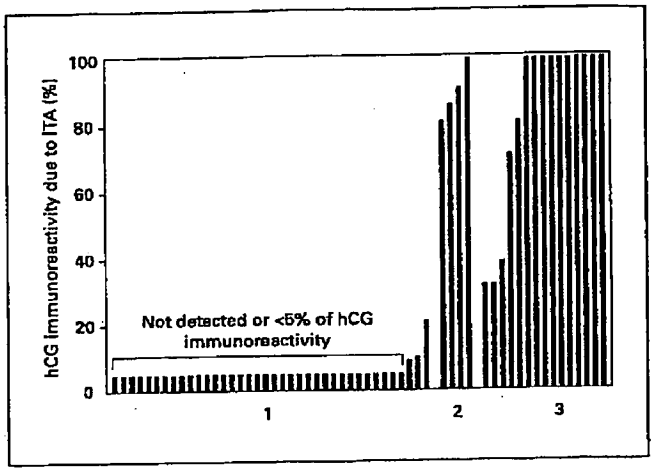

Fig. 11. Illustration of the sudden rise in measurement of core 2 hyperglycosylated forms of hCG (here termed 'ITA' and measured directly using C5 choriocarcinoma hCGn as standard) in three groups of patients being monitored for possible gestational trophoblastic disease. Group 1 includes patients without invasive disease but who continue to produce hCG at low but still abnormal concentrations after a pregnancy with a hydatidiform mole. Patients in group 1 produce little core 2 type hCG isoform (ITA). Group 2 are those patients from group 1 who suddenly begin to produce mostly hyperglycosylated hCG (core 2 hCG) indicating conversion of residual trophoblast cells to a more malignant state. Group 3 represents those patients with overt invasive choriocarcinoma. This study illustrates that the degree of malignancy may correlate with the production of these hCG isoforms. The sudden change to core 2 hCG isoforms as measured by B152 correlated with the sudden rise of hCG concentrations, especially in patients of group 2 who had earlier displayed very low detectable total hCG before a sudden rise in concentration that correlated to a sudden rise in core 2 hCG isoform or 'ITA' content. This figure is reprinted from Khanlian et al. [50] with permission of Elsevier, © 2003.

Conclusions

The fortuitous development of antibody B152 which recognizes one small linear region of hCGβ around residue 132, which contains a core 2 type of O-linked glycan instead of the usual predominant core 1 O-linked glycan present on the WHO standard mid-pregnancy isoform of hCG [46, 47], has led to the discovery of a number of clinical diagnostic uses for measurement of these 'hyperglycosylated' hCG isoforms. Apparently, these isoforms are naturally produced mainly in early pregnancy up to week 6 and are produced by cytotrophoblast cells leading one group to term the isoforms 'invasive trophoblastic antigen' or 'ITA'. A more appropriate nomenclature may

be the core 2-containing hCG isoforms, which appear to be very specifically measured by antibody B152. These isoforms have a major diagnostic application for patients with hCG-secreting cancers for which the degree of malignancy may be related to the percentage of this isoform produced by the malignant tissue. In addition, pregnancy-related applications include improved testing for Down syndrome and better prediction of the success of early pregnancy in fertility clinics. In summary, a whole previously unappreciated area of hCG diagnostic measurement has been opened by the development and application of the unique antibody B152 to quantify hCG isoforms which contain core 2 sugars on the COOH-terminal portion of the hCG β-subunit.

Acknowledgements

This review was supported by NIH grant R21 CA098350-02. My colleagues Dr. G. Kovalevskaya and Dr. J. F. O'Connor are acknowledged for providing helpful editorial advice.

References

1 Bahado-Singh R, Oz U, Shahabi S, Omrani A, Mahoney M, Cole L: Urine hyperglycosylated hCG plus ultrasound biometry for detection of down syndrome in the second trimester in a high-risk population. Obstet Gynecol 2000;95: 889–894.
2 Bahado-Singh RO, Oz U, Shahabi S, Mahoney MJ, Baumgarten A, Cole LA: Comparison of urinary hyperglycosylated human chorionic gonadotropin concentration with the serum triple screen for Down syndrome detection in high-risk pregnancies. Am J Obstet Gynecol 2000;183:1114–1118.
3 Cole LA, Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ: Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: A new basis for gestational Down syndrome screening. Clin Chem 1999;45:2109–2119.
4 Kovalevskaya G, Birken S, Kakuma T, O'Connor JF: Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. J Endocrinol 1999;161:99–106.
5 Kovalevskaya G, Genbacev O, Fisher SJ, Caceres E, O'Connor JF: Trophoblast origin of hCG isoforms: Cytotrophoblasts are the primary source of choriocarcinoma-like hCG. Mol Cell Endocrinol 2002;194:147–155.
6 Weinans MJ, Butler SA, Mantingh A, Cole LA: Urinary hyperglycosylated hCG in first trimester screening for chromosomal abnormalities. Prenat Diagn 2000;20:976–978.
7 Cole LA, Omrani A, Cermik D, Singh RO, Mahoney MJ: Hyperglycosylated hCG, a potential alternative to hCG in Down syndrome screening. Prenat Diagn 1998;18:926–933.
8 Cole LA, Shahabi S, Oz UA, et al: Urinary screening tests for fetal Down syndrome. 2. Hyperglycosylated hCG. Prenat Diagn 1999; 19:351–359.
9 Palomaki GE, Neveux LM, Knight GJ, Haddow JE, Pandian R: Maternal serum invasive trophoblast antigen (hyperglycosylated hCG) as a screening marker for Down syndrome during the second trimester. Clin Chem 2004;50: 1804–1808.
10 Palomaki GE, Knight GJ, Roberson MM, et al: Invasive trophoblast antigen (hyperglycosylated human chorionic gonadotropin) in second-trimester maternal urine as a marker for down syndrome: Preliminary results of an observational study on fresh samples. Clin Chem 2004;50:182–189.
11 Pandian R, Lu J, Ossolinska-Plewnia J: Fully automated chemiluminometric assay for hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen). Clin Chem 2003;49:808–810.
12 Pandian R, Cole LA, Palomaki GE: Second-trimester maternal serum invasive trophoblast antigen: A marker for Down syndrome screening. Clin Chem 2004;50:1433–1435.
13 Kovalevskaya G, Birken S, Kakuma T, et al: Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: Preliminary characterization of the hyperglycosylated hCG epitope. J Endocrinol 2002;172:497–506.
14 Cole LA, Sutton JM: Selecting an appropriate hCG test for managing gestational trophoblastic disease and cancer. J Reprod Med 2004;49: 545–553.
15 Sutton JM, Cole LA: Sialic acid-deficient invasive trophoblast antigen (sd-ITA): A new urinary variant for gestational Down syndrome screening. Prenat Diagn 2004;24:194–197.
16 Cole LA, Sutton JM: HCG tests in the management of gestational trophoblastic diseases. Clin Obstet Gynecol 2003;46:523–540.
17 Berger P, Sturgeon C, Bidart JM, et al: The ISOBM TD-7 Workshop on hCG and related molecules. Towards user-oriented standardization of pregnancy and tumor diagnosis: Assignment of epitopes to the three-dimensional structure of diagnostically and commercially relevant monoclonal antibodies directed against human chorionic gonadotropin and derivatives. Tumor Biol 2002;23:1–38.
18 Rye PD, Nustad K, Stigbrand T: Tumor marker workshops. Tumor Biol 2003;24:165–171.
19 Butler SA, Iles RK: The free monomeric beta subunit of human chorionic gonadotrophin (hCG beta) and the recently identified homodimeric beta-beta subunit (hCG beta beta) both have autocrine growth effects. Tumor Biol 2004;25:18–23.
20 Matsui H, Iitsuka Y, Yamazawa K, et al: Criteria for initiating chemotherapy in patients after evacuation of hydatidiform mole. Tumor Biol 2003;24:140–146.
21 Steinmeyer C, Berkholz A, Gebauer G, Jager W: The expression of hCG receptor mRNA in four human ovarian cancer cell lines varies considerably under different experimental conditions. Tumor Biol 2003;24:13–22.
22 Stenman UH, Alfthan H, Hotakainen K: Human chorionic gonadotropin in cancer. Clin Biochem 2004;37:549–561.
23 Birken S, Krichevsky A, O'Connor J, et al: Development and characterization of antibodies to a nicked and hyperglycosylated form of hCG from a choriocarcinoma patient: Generation of antibodies that differentiate between pregnancy hCG and choriocarcinoma hCG. Endocrine 1999;10:137–144.
24 Cole LA, Khanlian SA, Sutton JM, Davies S, Stephens ND: Hyperglycosylated hCG (invasive trophoblast antigen, ITA) a key antigen for early pregnancy detection. Clin Biochem 2003; 36:647–655.
25 Birken S, Gawinowicz MA, Kardana A, Cole LA: The heterogeneity of human chorionic gonadotropin (hCG). 2. Characteristics and origins of nicks in hCG reference standards. Endocrinology 1991;129:1551–1558.
26 Birken S, Chen Y, Gawinowicz MA, et al: Separation of nicked human chorionic gonadotropin (hCG), intact hCG, and hCG beta fragment from standard reference preparations and raw urine samples. Endocrinology 1993; 133:1390–1397.
27 Cole LA, Kardana A, Andrade-Gordon P, et al: The heterogeneity of human chorionic gonadotropin (hCG). 3. The occurrence and biological and immunological activities of nicked hCG. Endocrinology 1991;129:1559–1567.

28 Lee IS, Chung DY, Cole LA, Copel JA, Isozaki T, Hsu CD: Elevated serum nicked and urinary beta-core fragment hCG in preeclamptic pregnancies. Obstet Gynecol 1997;90:889–892.

29 Cole LA, Kardana A, Park SY, Braunstein GD: The deactivation of hCG by nicking and dissociation. J Clin Endocrinol Metab 1993;76:704–710.

30 Puisieux A, Bellet D, Troalen F, et al: Occurrence of fragmentation of free and combined forms of the beta-subunit of human chorionic gonadotropin. Endocrinology 1990;126:687–694.

31 Nishimura R, Kitajima T, Hasegawa K, Takeuchi K, Mochizuki M: Molecular forms of human chorionic gonadotropin in choriocarcinoma serum and urine. Jpn J Cancer Res 1989;80:968–974.

32 Birken S, Kovalevskaya G, O'Connor J: Metabolism of hCG and hLH to multiple urinary forms. Mol Cell Endocrinol 1996;125:121–131.

33 Birken S, Agosto G, Amr S, et al: Characterization of antisera distinguishing carbohydrate structures in the beta-carboxyl-terminal region of human chorionic gonadotropin. Endocrinology 1988;122:2054–2063.

34 Amr S, Rosa C, Birken S, Canfield R, Nisula B: Carboxyterminal peptide fragments of the beta subunit are urinary products of the metabolism of desialylated human choriogonadotropin. J Clin Invest 1985;76:350–356.

35 Birken S, Canfield R, Agosto G, Lewis J: Preparation and characterization of an improved beta-COOH-terminal immunogen for generation of specific and sensitive antisera to human chorionic gonadotropin. Endocrinology 1982;110:1555–1563.

36 Birken S, Armstrong EG, Kolks MA, et al: Structure of the human chorionic gonadotropin beta-subunit fragment from pregnancy urine. Endocrinology 1988;123:572–583.

37 Elliott MM, Kardana A, Lustbader JW, Cole LA: Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine 1997;7:15–32.

38 Brockhausen I: Biosynthesis and functions of O-glycans and regulation of mucin antigen expression in cancer. Biochem Soc Trans 1997;25:871–874.

39 Brockhausen I, Schutzbach J, Kuhns W: Glycoproteins and their relationship to human disease. Acta Anat (Basel) 1998;161:36–78.

40 Brockhausen I: Pathways of O-glycan biosynthesis in cancer cells. Biochim Biophys Acta 1999;1473:67–95.

41 Amano J, Nishimura R, Mochizuki M, Kobata A: Comparative study of the mucin-type sugar chains of human chorionic gonadotropin present in the urine of patients with trophoblastic diseases and healthy pregnant women. J Biol Chem 1988;263:1157–1165.

42 Krichevsky A, Birken S, O'Connor J, et al: Development, characterization, and application of monoclonal antibodies to the native and synthetic beta COOH-terminal portion of human chorionic gonadotropin (hCG) that distinguish between the native and desialylated forms of hCG. Endocrinology 1994;134:1139–1145.

43 Birken S, Yershova O, Myers RV, Bernard MP, Moyle W: Analysis of human choriogonadotropin core 2 O-glycan isoforms. Mol Cell Endocrinol 2003;204:21–30.

44 Birken S, Maydelman Y, Gawinowicz MA, Pound A, Liu Y, Hartree AS: Isolation and characterization of human pituitary chorionic gonadotropin. Endocrinology 1996;137:1402–1411.

45 O'Connor JF, Ellish N, Kakuma T, Schlatterer J, Kovalevskaya G: Differential urinary gonadotrophin profiles in early pregnancy and early pregnancy loss. Prenat Diagn 1998;18:1232–1240.

46 Bristow A, Berger P, Bidart JM, et al: Establishment, value assignment, and characterization of new WHO reference reagents for six molecular forms of human chorionic gonadotropin. Clin Chem 2005;51:177–182.

47 Birken S, Berger P, Bidart JM, et al: Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites. Clin Chem 2003;49:144–154.

48 Pidoux G, Guibourdenche J, Frendo JL, et al: Impact of trisomy 21 on human trophoblast behaviour and hormonal function. Placenta 2004;25(suppl A):S79–S84.

49 Frendo JL, Guibourdenche J, Pidoux G, et al: Trophoblast production of a weakly bioactive human chorionic gonadotropin in trisomy 21-affected pregnancy. J Clin Endocrinol Metab 2004;89:727–732.

50 Khanlian SA, Smith HO, Cole LA: Persistent low levels of human chorionic gonadotropin: A premalignant gestational trophoblastic disease. Am J Obstet Gynecol 2003;188:1254–1259.

51 Lapthorn AJ, Harris DC, Littlejohn A, et al: Crystal structure of human chorionic gonadotropin. Nature 1994;369:455–461.

52 Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA: Structure of human chorionic gonadotropin at 2.6. A resolution from MAD analysis of the selenomethionyl protein. Structure 1994;2:545–558.